At least one, and probably more than one, α helix of domain 1 is involved in the formation of ion channels and pores within the insect midgut epithelium (Gazit and Shai, 1993; Gazit and Shai, 1995). Rather than target for mutagenesis the sequences encoding the α helices of domain 1 as others have (Wu and Aronson, 1992; Aronson *et al.*, 1995; Chen *et al.*, 1995), the present inventors opted to target exclusively sequences encoding amino acid residues adjacent to or lying within the predicted loop regions of Cry1C that separate these α helices. Amino acid residues within these loop regions or amino acid residues capping the end of an α helix and lying adjacent to these loop regions may affect the spatial relationships among these α helices. Consequently, the substitution of these amino acid residues may result in subtle changes in tertiary structure, or even quaternary structure, that positively impact the function of the ion channel. Amino acid residues in the loop regions of domain 1 are exposed to the solvent and thus are available for various molecular interactions. Altering these amino acids could result in greater stability of the protein by eliminating or occluding protease-sensitive sites. Amino acid substitutions that change the surface charge of domain 1 could alter ion channel efficiency or alter interactions with the brush border membrane or with other portions of the toxin molecule, allowing binding or insertion to be more effective.

In mutating specific residues within these loop regions, the inventors were able to produce synthetic crystal proteins which retained or even enhanced insecticidal activity against lepidopteran insects.

According to this invention, base substitutions are made in *cry1C* codons in order to change the particular codons with the loop regions of the polypeptides, and particularly, in those loop regions between α-helices. As an illustrative embodiment, changes in three such amino acids within the loop region between α-helices 3 and 4 of domain 1 produced modified crystal proteins with enhanced insecticidal activity.

The insecticidal activity of a crystal protein ultimately dictates the level of crystal protein required for effective insect control. The potency of an insecticidal protein should be maximized as much as possible in order to provide for its economic and efficient utilization in the field. The increased potency of an insecticidal protein in a bioinsecticide formulation would be expected to improve the field performance of the bioinsecticide

product. Alternatively, increased potency of an insecticidal protein in a bioinsecticide formulation may promote use of reduced amounts of bioinsecticide per unit area of treated crop, thereby allowing for more cost-effective use of the bioinsecticide product. When expressed *in planta*, the production of crystal proteins with improved insecticidal activity can be expected to improve plant resistance to susceptible insect pests.

The most effective crystal protein against the beet armyworm, *Spodoptera exigua*, is the Cry1C protein, yet the toxicity of this toxin towards *S. exigua* is ~40-fold less than the toxicity of Cry1Ac towards the tobacco budworm, *Heliothis virescens*, and ~50-fold less than the toxicity of Cry1Ba towards the diamondback moth, *Plutella xylostella* (Lambert *et al.*, 1996). Accordingly, there is a need to improve the toxicity of Cry1C towards *S. exigua* as well as towards other lepidopteran pests. Previously, site-directed mutagenesis was used to probe the function of two surface-exposed loop regions found in domain 2 of the Cry1C protein (Smith and Ellar, 1994). Although amino acid substitutions within domain 2 were found to affect insecticidal specificity, Cry1C mutants with improved insecticidal activity were not obtained.

In sharp contrast to the prior art which has focused on generating amino acid substitutions *within* the predicted α-helices of domain 1 in Cry1A, the novel mutagenesis strategies of the present invention focus on generating amino acid substitutions at positions *near or within the predicted loop regions* connecting the α-helices of domain 1. These loop regions are shown in the schematic of crystal protein domains shown in FIG. 1. In mutating specific residues within these loop regions, the inventors were able to produce synthetic crystal proteins which retained or possessed enhanced insecticidal activity against certain lepidopteran pests, including the beet armyworm, *S. exigua*.

According to this invention, base substitutions are made in *cry1C* codons in order to change the particular codons encoding amino acids within or near the predicted loop regions between the α-helices of domain 1. As an illustrative embodiment, changes in three such amino acids within the loop region between α-helices 3 and 4 of domain 1 produced modified crystal proteins with enhanced insecticidal activity (Cry1C.499, Cry1C.563, Cry1C.579). As a second illustrative embodiment, an alanine substitution for an arginine residue within or adjacent to the loop region between α-helices 4 and 5

produced a modified crystal protein with enhanced insecticidal activity (Cry1C-R148A). Although this substitution removes a potential trypsin-cleavage site within domain 1, trypsin digestion of this modified crystal protein revealed no difference in proteolytic stability from the native Cry1C protein. Furthermore, the R180A substitution in Cry1C (Cry1C-R180A) also removes a potential trypsin cleavage site in domain 1, yet this substitution has no effect on insecticidal activity. Thus, the steps in the Cry1C protein mode-of-action impacted by these amino acid substitutions have not been determined nor is it obvious what substitutions need to be made to improve insecticidal activity.

Many crystal proteins show significant amino acid sequence identity to the Cry1C amino acid sequence within domain 1, including proteins of the Cry1, Cry2, Cry3, Cry4, Cry5, Cry7, Cry8, Cry9, Cry10, Cry11, Cry12, Cry13, Cry14, and Cry16 classes defined by the new *cry* gene nomenclature (Table 1). Furthermore, the structures for CryIIIA (Cry3A) and CryIAa (Cry1Aa) show a remarkable conservation of protein tertiary structure (Grochulski *et al.*, 1995). Thus, it is anticipated that the mutagenesis of codons encoding amino acids within or near the loop regions between the α-helices of domain 1 of these proteins may also result in the generation of improved insecticidal proteins. Indeed, an alignment of Cry1 amino acid sequences spanning the loop region between α-helices 4 and 5 reveals that several Cry1 proteins contain an arginine residue at the position homologous to R148 of Cry1C. Since the Cry1C R148A mutant exhibits improved toxicity towards a number of lepidopteran pests, the inventors contemplate that similar substitutions in these other Cry1 proteins will also yield improved insecticidal proteins.

4.2 METHODS FOR PRODUCING CRY1C* PROTEINS

The *B. thuringiensis* strains described herein may be cultured using standard known media and fermentation techniques. Upon completion of the fermentation cycle, the bacteria may be harvested by first separating the *B. thuringiensis* spores and crystals from the fermentation broth by means well known in the art. The recovered *B. thuringiensis* spores and crystals can be formulated into a wettable powder, a liquid concentrate, granules or other formulations by the addition of surfactants, dispersants,